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An electron microscope study of the proteins attached to polio virus RNA and its replicative form (RF)

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### ABSTRACT

*A recently described method (Wu, M. and Davidson, N. (1978), Nucleic Acids Research 5, in press) for visualizing proteins attached to nucleic acids in the electron microscope has been applied to study proteins attached to poliovirion RNA and to the viral double-stranded intracellular RF form. A protein is found at the 5' end of the plus strand virion RNA, and protein components are found at both ends of the duplex RF. In the RF as usually extracted, there is frequently a larger or compound protein aggregate at the end which contains the 3' end of the plus strand and the 5' end of the minus strand. Banding in CsCl-guanidinium hydrochloride in the presence of sarkosyl causes dissociation of some components of this aggregate, leaving both ends labeled with the covalently bound VPg. These results confirm and extend previous biochemical studies of proteins bound to poliovirion RNA and to the RF form.*

### INTRODUCTION

Biochemical studies show that there is a protein, VPg, of molecular weight approximately 6,000 Daltons covalently attached to the 5' end of poliovirion RNA (1,2,3). An apparently identical protein is found covalently attached to the 5' poly(U) end of the minus strand RNA in the intracellular double stranded polio replicative form (RF) (3) and to the nascent plus strands in the replicative intermediate (RI) (2,3). We report here our studies of poliovirion RNA and of the RF form by a newly developed electron microscope method for visualizing proteins attached to nucleic acids (4). These results confirm and extend the biochemical studies and further suggest that, in the RF as usually extracted, there are additional protein components non-covalently attached to that end of the RF which contains the 3' end of the virion RNA and the 5' end of the minus strand.

The electron microscope labeling method of Wu and Davidson (4) involves treatment of the protein-nucleic acid complexes with dinitrofluorobenzene (DNFB), thus selectively labeling the protein with dinitrophenyl (DNP)

groups. The  $(\text{DNP})_n$ -protein-nucleic acid complex is then treated with rabbit anti-DNP IgG. The antibody DNP aggregate on the nucleic acid strand can be visualized by protein free spreading methods and, if the aggregate is large enough, in cytochrome-c spreads. The size of the primary antibody DNP aggregate can be amplified by further treatment with goat anti-rabbit IgG. In all cases studied the aggregate after treatment with the second antibody is large enough to be visualized in conventional cytochrome-c spreads.

In the previous tests, the method was used to label proteins of molecular weight 50,000 or greater, attached to single- or double stranded DNA. It is a matter of considerable technical interest to determine whether the method is effective for visualizing much smaller proteins, such as VPg, and whether the immunologic reagents used can be purified sufficiently so that difficulties due to RNAase action will not be encountered in mapping proteins attached to RNA.

### MATERIALS AND METHODS

Polio RNA Samples. Polio virus was grown and virion RNA extracted, essentially as previously described (5,6). This procedure involves lysis of virions with phenol-chloroform in 0.5% SDS, and centrifugation of the RNA through a 10-30% sucrose gradient in 0.1 M NaCl, 0.01 M Tris (pH 7.5), 2 mM EDTA, 0.01 M mercaptoethanol, 0.5% SDS, followed by ethanol precipitation.

Polio RF was extracted from infected cells as previously described (7). It should be noted that this preparation involves deproteinization with phenol-chloroform-isopentanol in 0.5% SDS, and gel filtration through Sepharose 2B in 0.1 M NaCl, 0.01 M Tris·HCl (pH 7.5), 2 mM EDTA, 0.5% SDS.

Labeling of polio protein-nucleic acid complexes for electron microscopic visualization. Unless otherwise specified, sources of reagents and purification were as previously described (4). The following modifications in the procedure have been introduced. To minimize aggregation, the monovalent Fab fragment of goat anti-rabbit IgG was used as a second antibody stain. The monovalent Fab fragment was prepared from purified goat anti-rabbit IgG according to Nisonoff, et al. (8). The incubation times with anti-DNP IgG and with Fab were decreased, and consisted of preincubation at 0° for 10 min followed by incubation for 15 min at 37°. The bound IgG or Fab was then crosslinked to the DNP-protein-nucleic acid complex by ultraviolet irradiation, followed by separation from unbound IgG or Fab with a Sepharose 2B column. The uv crosslinking replaces glutaraldehyde treatment.

We believe that uv crosslinking is preferable to the glutaraldehyde

treatment used previously for several reasons. It is more convenient, and the crosslinks are more stable towards dissociation. With high concentrations of glutaraldehyde, protein-protein crosslinking causes precipitation. With lower concentrations, some protein-nucleic acid complexes dissociate upon standing. The antigenic determinants of the rabbit IgG do not appear to be inactivated by the irradiation.

In a typical experiment, one  $\mu\text{g}$  of RNA was dissolved in 20  $\mu\text{l}$  of a solution containing 0.125 M  $\text{Na}_2\text{B}_4\text{O}_7$  buffer (pH 9.0), 6.25 mM  $\text{Na}_3\text{EDTA}$  and 10% v/v dimethyl sulfoxide (BED buffer). Five  $\mu\text{l}$  of 0.21 M DNFB stock solution in absolute ethanol was added with gentle mixing. The reaction mixture was incubated at 37° in the dark for one hour.

Unreacted DNFB was removed by several ether extractions. Hydrolyzed DNFB (dinitrophenol) was removed by dialysis against 2 changes of 10 mM  $\text{Na}_3\text{EDTA}$ , 0.4 M NaCl, 0.1 M Tricine buffer (pH 8.0) (2X ENTc buffer) for 2 hours each at 4°C. An equal volume of 10 mg/ml of rabbit anti-DNP IgG in 0.01 M borate buffer (pH 8.0) was added and mixed gently. The mixture was kept at 0°C for 10 min then incubated at 37°C for 15 min. The mixture was then transferred onto a clean Teflon block as 20-30  $\mu\text{l}$  droplets and irradiated at a distance of 10 cm from a General Electric G15T8 15 watt germicidal lamp for 5 min. The radiation dose is approximately 700  $\mu\text{watt cm}^{-2}$ . Test experiments showed that this was approximately the optimal condition for maximal crosslinking and minimum strand breakage.

Unbound rabbit anti-DNP IgG was removed by gel filtration through a 0.5 ml Sepharose 2B column equilibrated with ENTc buffer. 5  $\mu\text{l}$  of eluted RNA-IgG complex was diluted onto 100  $\mu\text{l}$  of cytochrome-c formamide spreading solution for electron microscopy. The remainder was either mixed with an equal volume of 10 mg/ml monovalent Fab fragment of goat anti-rabbit IgG or precipitated with ethanol to concentrate and store for later reaction with Fab. Incubation with Fab, uv crosslinking, and Sepharose 2B chromatography were carried out as described above.

Identification of the 3' poly(A) ends of RNA molecules. 3' poly(A) ends of single-stranded or double-stranded RNA's were labeled by hybridization to poly(dBrU) sequences of length approximately 300 nucleotides polymerized onto the 800 base pair circles of trypanosome mitochondrial DNA, as described by Bender and Davidson (9) and by Bender *et al.* (10). The trypanosome circular DNA preparation was a gift from Dr. L. Simpson; the dBrU tails were added by Dr. W. Bender. 0.5  $\mu\text{g}$  of DNFB treated single stranded polio virion RNA or double stranded RF was incubated with the label at a molar ratio of 1

to 5 (estimated by electron microscopy) in 10  $\mu$ l of 0.1 M sodium phosphate (NaP) buffer (pH 7.0), 0.01 M NaCl at room temperature for 10 min before further reaction.

After reaction with the trypanosome circles, reaction with rabbit anti-DNP IgG and monovalent Fab were carried out in 10 mM Na<sub>2</sub>EDTA, 0.2 M NaCl, 0.1 M NaP buffer (pH 7.2) (ENP buffer) instead of ENTc buffer. The Sepharose 2B column used to remove excess IgG was also equilibrated in ENP buffer.

Further purification of polio RF and denaturation of polio RF. For some experiments, polio RF was further purified by banding in CsCl-guanidinium hydrochloride (GuHCl) gradients according to Enea and Zinder (11) as modified by Dr. Peter Chandler (personal communication). A 5 ml solution containing 6.44 g CsCl, 1.2 g GuHCl, 0.05 M phosphate buffer (pH 7.2), 0.05 M Na<sub>2</sub>EDTA, 0.2% sarkosyl, and 5  $\mu$ g polio [<sup>3</sup>H]RF was centrifuged at 35K at 20°C for 65 hours in a Beckman SW 50.1 rotor. The fractions containing [<sup>3</sup>H] RNA were collected, pooled, diluted 2.5 fold with 0.01 M phosphate buffer, and precipitated with ethanol.

Polio RF was denatured by heating to 100°C for 2 min in 1 mM Na<sub>2</sub>EDTA (pH 7.5) (12).

Electron Microscopy. Basic protein film spreading with cytochrome-c was carried out according to Davis *et al.* (13). The spreading solution contained 55% formamide, 0.06 M Tris (pH 8.2), 6 mM EDTA; the hypophase was 15% formamide, 0.01 M Tris (pH 8.2), 1 mM EDTA. Benzyldimethylalkyl ammonium chloride (BAC) spreading was a modification of the procedure of Vollenweider *et al.* (14, 4). The spreading solution was 50% urea-formamide (15), 10 mM triethanolamine buffer (pH 7.5), 0.5% HCHO. The hypophase was redistilled H<sub>2</sub>O.

In the various experiments reported in the Results sections, about 30-40% of the single-stranded RNA and 80-85% of the double-stranded RF was judged to be full or close to full length by visual inspection. The molecular lengths of polio virus single-strand RNA and of the duplex RF were measured relative to  $\phi$ X-174 single strands (5.38 kb) and SV40 duplex circles (5.23 kb) as  $7.7 \pm 0.8$  and  $7.5 \pm 0.5$  kb, respectively. In experiments in which frequencies of labeling of full length molecules were scored, all long molecules in random scans of the EM grids were photographed. Those with measured lengths within  $\pm 10\%$  of the length given above were included in the sample.

## RESULTS AND DISCUSSION

Visualization of the protein (VPg) attached to polio virus genome RNA and confirmation of its location at the 5' end. Poliovirus genome RNA was treated with DNFB and incubated with poly (dBrU) tailed trypanosome mitochondrial DNA circles as described in Materials and Methods. Samples were mounted for electron microscopy by both BAC and cytochrome-c spreading methods. Trypanosome circles were seen attached to one end of many molecules. Quantitative data were collected from one cytochrome-c spread. 18 out of 50 full length RNA strands has a trypanosome circle at one end. No dots that could be interpreted as a protein were seen at the ends of molecules in either BAC or cytochrome-c spreads. The preparation was then treated with rabbit anti-DNP IgG in ENP buffer and irradiated with uv light. A clear dot was then observed at one end of many molecules in BAC spreads (Fig. 1a). However, no dots were visible in cytochrome-c spreads. A sample of 50 intact single-stranded RNA molecules was studied by the BAC method. 34 molecules had a dot at one end, 15 molecules did not have a terminal dot, and one molecule had dots at both ends. 16 molecules had their poly(A) ends labeled with a trypanosome circle; of these, 10 had a terminal dot at the other end. There were no molecules with the trypanosome circle and the dot at the same

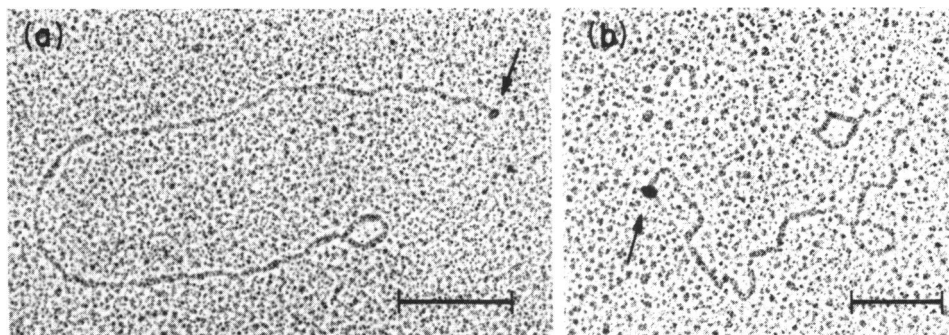


Fig. 1. a) Electron micrograph of poliovirion RNA spread by the BAC method after treatment with DNFB and rabbit anti-DNP IgG, and with the 3' poly(A) end labeled with trypanosome circles tailed with poly(dBrU). There is a terminal protein dot at the 5' end (arrow). b) A cytochrome-c spread of poliovirion RNA treated with DNFB, anti-DNP, and the Fab fragment of goat anti-rabbit IgG, and with the 3' poly(A) end labeled by a trypanosome circle. An arrow points to the terminal protein dots. The bar represents 1 kb.

end. There were 9 molecules with neither dot nor circle. Various secondary structure features in single stranded RNA were observed under the spreading conditions used, but have not been quantitatively mapped. No circular molecules were observed.

After reaction with the monovalent Fab fragments, a large dot was clearly observed in many molecules with cytochrome-c spreading. 13 of 30 intact single-stranded molecules had a dot at one end. 8 of the 30 molecules had a small circle at one end. 5 molecules had a dot at one end and a small circle at the other (Fig. 1b). No molecules with an internal dot were observed.

In control experiments with ribosomal RNA, dots were not seen at the ends of molecules after treatment with DNFB and one or both antibodies with either spreading method. Similar control experiments with single- and double-stranded DNA were carried out in our previous study (4).

These experiments show that there is a molecule which reacts with DNFB-- presumably a protein-- at one end of polio virus genome RNA. The poly(A) labeling experiments show that the reactive molecule is at the 5' end and we identify it as VPg. The amount of rabbit anti-DNP IgG bound to the end is sufficient so that the aggregate can be seen by the high resolution BAC spreading method, but not by cytochrome-c spreading. After amplification with goat anti-rabbit Fab fragments, the aggregate is large enough to be seen in cytochrome-c spreads. Similar observations were made for some DNA- protein complexes previously (4).

In the quantitative data reported above for molecules labeled with a single antibody and trypanosome circles and observed by BAC spreads, the efficiency of poly(A) end labeling was 32%, the efficiency of labeling the terminal protein was 68%; the observed efficiency of labeling both poly(A) and protein ends was 20%, in agreement with a prediction of 22% from the above data.

After additional labeling with the Fab fragment and cytochrome-c spreads, the efficiencies of poly(A) and terminal protein labeling were 27% and 43% respectively. The observed efficiency for appropriate double labeling was 17%, in agreement with the predicted value of 11%.

### Visualization of Proteins on Polio RF

a) IgG clusters can be observed at both ends of polio RF; the cluster at the poly(A) end is frequently larger and more complex than the other. Polio RF was treated with DNFB, rabbit anti-DNP IgG, and the Fab fragment of goat anti-rabbit IgG. Some circular duplex RNA molecules were observed; these are discussed in a later section.

In linear RF molecules labeled with the first antibody, the terminal dot was recognizable in BAC spreads, but it was more difficult to recognize than was the terminal dot on single-stranded RNA. Just as for the single strands, dots were readily observed in cytochrome-c spreads in molecules treated with the Fab fragment as a second antibody. Several types of molecules were observed in such preparations. About 19% were linear with no visible dots and 81% had one of the several kinds of terminal dot configurations described below. Two different kinds of protein aggregates or dots were observed at the ends of the molecules. Some were simple approximately round dots of approximately the same size as observed for single-stranded RNA. Other dots were larger, and were either asymmetric in shape or appeared as two or three resolved but adjacent dots of approximately equal size. We refer to these structures as simple and compound dots, respectively. Linear RF molecules with a simple dot, a double dot and a triple dot at one end are shown in Figs. 2a, 2b, and 2c, respectively. Fig. 2d and 2e show linear molecules with a simple dot at one end and a compound dot at the other (2d); and simple dots at both ends (2e).

Statistical data on the several kinds of structures are given in Table I. 16% and 25% of all molecules had only one end labeled with a simple or a compound dot, respectively. 5.5% of all molecules had both ends labeled with a simple dot, and 11% of all molecules had one simple dot and one compound dot. No molecules with compound dots at both ends were observed.

In order to determine the polarity of the asymmetrical labeling, polio RF samples were treated with DNFB, and hybridized to trypanosome-poly(dBrU) circles. This procedure is possible because the 3'-terminal poly(A) of plus strands in polio RF is 2-3 times longer than the complementary 5'-terminal poly(U) of minus strands (7.16). Therefore, a single-stranded poly(A) tail protrudes out of the double-stranded RNA. (We wish to note, however, that A:BrU base pairs are more stable than A:U base pairs. It is conceivable that poly(dBrU) will displace the poly(U) from a completely matched A:U duplex segment.) 23 out of 100 intact monomer linear polio RF molecules had the small trypanosome circle attached at one end (Fig. 2f). This preparation was then treated with the first and second antibodies and spread for electron microscopy in cytochrome-c films as described in Materials and Methods. Among 200 intact linear monomers, 41 had trypanosome circles attached at one end. 8 of these 41 molecules had both ends labelled with an IgG cluster; 5 of these molecules had asymmetrical labeling. In all five, the compound dot was present at the poly(A) end and the opposite end had the simple dot (Fig.

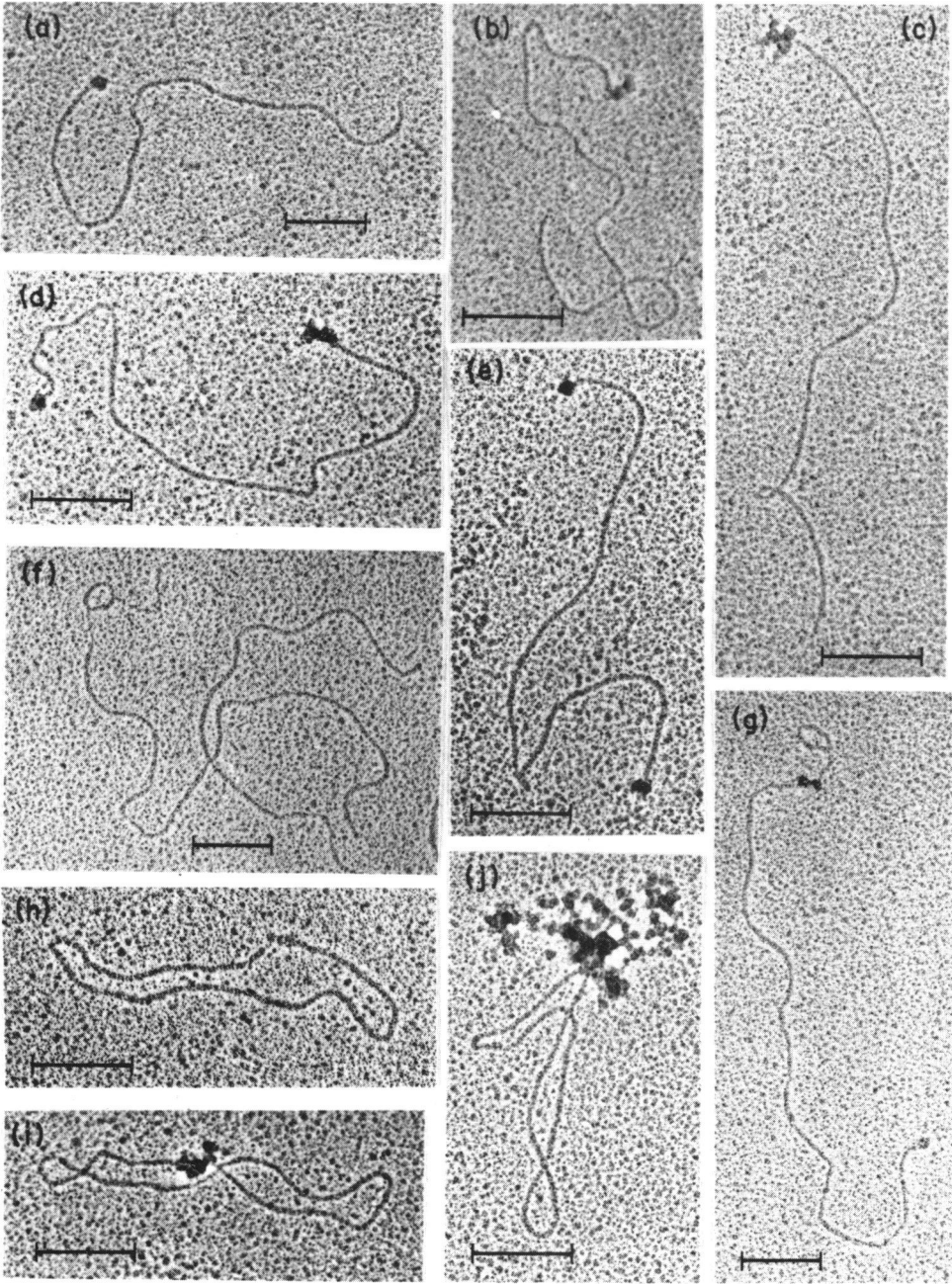




Table 1  
Frequency of observed labeling of polio RF VPg\*

Preparation:	Linear monomers with no dot	Linear monomers with one end labeled			Linear monomers with both ends labeled			Circles with protein linkage	Total
		Single dot	Compound dot	Total	Symmetrical	Asymmetrical	Total		
Before GuHCl-CsCl-sarkosyl gradient	38	32	50	82	11	22	33	47	200
After GuHCl-CsCl-sarkosyl gradient	35	85	4	89	29	3	32	44	200

\*Cytochrome-c spreads of molecules treated with DNFB and both antibodies.

2g). 17 of the 41 poly(A) labeled molecules had one end labeled with a dot. Among these, 6 had the non-poly(A) end labeled with a simple dot, 5 had the poly(A) end labeled with a simple dot and 6 had the poly(A) end labeled with a compound dot.

The totality of the data presented above are consistent with the model that there is protein at both ends of polio RF, that the protein at the 5' end of the plus strand has the same size as that observed for single-stranded genome RNA, but that, in the sample as extracted, there is frequently (60-70% of the sample) a more complex structure at that RF end which corresponds to the 5' poly(U) terminus of the minus strand and the 3' poly(A) end of the plus strand.

b) The compound cluster is dissociated by banding in CsCl-GuHCl-sarkosyl gradients, and by heat denaturation of the RF. 5 µg of polio RF was subjected to further purification by banding in CsCl-GuHCl-sarkosyl gradients as described in Materials and Methods. 3.2 µg of RNA was recovered from a broad band about one quarter of the distance from the bottom of the centrifuge

Fig. 2. Cytochrome-c spreads of polio RF. All molecules have been treated with DNFB. Unless otherwise specified, molecules have been treated with anti-DNP, and goat anti-rabbit Fab. Molecules are shown with: a) a simple dot at one end; b) a double dot at an end; c) a triple dot at an end; d) a simple dot at one end and a compound dot at the other; e) a simple dot at each end; f) this molecule has its poly(A) end labeled with a trypanosome circle, but has not been treated with either antibody; g) a molecule treated with both antibodies and with the poly(A) label, showing a compound dot and a trypanosome circle at one end and a simple dot at the other; h) a circular molecule from a sample treated with anti-DNP only; i) a circular molecule after treatment with both antibodies; there is a clear indication of a protein aggregate at the junction of the two ends; j) a circular molecule after treatment with anti-DNP and goat anti-rabbit IgG (instead of the Fab fragment), showing a large aggregate of rabbit IgG and goat anti-rabbit IgG.

Each bar represents 1 kb.

tube. This RNA was concentrated, treated with DNFB, labeled with both antibodies, and mounted for electron microscopy in a cytochrome-c film. The results are summarized in the second line of Table 1. The frequency of molecules labeled at both ends is not affected, but the frequency of compound dots is greatly reduced. The simple dot structures appeared similar to those shown in Fig. 2e.

0.25  $\mu$ g of polio RF was denatured by heating to 100°C for two minutes in 20  $\mu$ l of 1 mM Na<sub>2</sub>EDTA (pH 7.5) before labeling. In an inspection in the electron microscope of 50 intact single-stranded molecules, 15 had no terminal dot, 34 had a simple dot attached at one end, and one had a compound (double) dot attached at one end. None had dots attached to both ends.

The frequency of end labeling with dots is sufficiently high to show that the 5' ends of both plus and minus strands are labeled, but some component of the compound dots is dissociated by the heat treatment and they are converted into simple dots.

c) Modification of the terminal proteins of RF induce circularization. Circular molecules were seen in the RF preparation with increasing frequency for each step in the labeling procedure. These data are presented in Table 2. The fraction of circular molecules increased from 1% to 5% to 13% to 22% with dinitrophenylation, and incubation with the first and second antibodies, respectively.

Fig. 2h is a circular molecule after treatment with the first antibody as observed in a cytochrome-c spread. Comparison with circular molecules observed after only DNFB treatment indicates that one cannot positively identify the protein junction where the two ends are joined in Fig. 2h. A protein aggregate identifying the junction is seen very clearly in most circular molecules formed after incubation with both antibodies (Fig. 2i).

These observations show that attachment of DNFB, and of the first and second antibodies to the terminal proteins of the RF successively increase a cohesive interaction between the ends.

d) On the usefulness of monovalent (Fab) fragments. In the initial stages of the present study, when we used goat anti-rabbit IgG as the second antibody, we frequently observed large protein aggregates at the end of a linear RNA or at one point, presumably the cohesive junction of the two ends, on circular molecules. Such an aggregate on a circular RF molecule is shown in Fig. 2j. We presume that this is an aggregate of multiple antigen and antibody molecules due to the presence of some remaining rabbit IgG in the solution despite the gel filtration step after the first antibody staining and

Table 2

Frequencies of circularization of polio RF after different treatments.

Treatment	No. of linear monomers	No. of linear* oligomers	Monomer circles		Total
			No.	%	
Control	192	6	2	1	200
DNFB	185	5	10	5	200
DNFB and rabbit anti-DNP	162	12	26	13	200
DNFB, anti-DNP, and goat anti-rabbit Fab	147	9	44	22	200

\*This class of molecules might contain some DNA contaminants.

uv crosslinking. The problem of large antigen-antibody aggregates is solved by using the Fab fragments.

#### FURTHER DISCUSSION

The first points are that the protein labeling procedure previously described (4) is effective for labeling the 6000 dalton polio VPg protein for electron microscopic observation and that the immunological reagents used are sufficiently free of RNAase so that mapping of proteins on single-stranded RNA is possible. In this labeling method, DNP groups become attached to nucleophilic groups, presumably mainly exposed ones and presumably mainly lysines, on the protein. The size of the aggregate after treatment with one or both antibodies is presumably related to the number of bound DNP groups. The polar VPg is positively charged at pH 7.5 (3), but the amino acid composition and the number of groups potentially reactive with DNFB are not known. Nevertheless, it is satisfying that the protein can be labeled and identified at a frequency of about 68% on single-stranded polio RNA. If circular molecules are scored as having both ends labeled, the data in Table 1 indicate that 60% of the ends of polio RF are identifiably labeled by this procedure.

Our observations confirm the previous biochemical studies in showing that there is a protein at the 5' end of the single-stranded virion RNA (1,2). Previous biochemical studies show that there is a protein attached to the 5' (poly (U)) end of minus strand RNA in RF (3). The nascent plus strands of the replicative intermediate have VPg at their 5' end (2,3). It was there-

fore a highly probable inference that there is also a protein at the 5' end of the plus strand in RF, but this had not been shown experimentally. Our studies show that both ends of the RF do indeed have a protein. We further observe that there are "compound" dots at the poly(A) end of RF as normally extracted. The compound dots are converted into simple dots--presumably VPg--by banding in CsCl-GuHCl-sarkosyl, or by heat.

These results suggest that in addition to the VPg protein covalently attached to the 5' end of the minus strand in the RF there is some non-covalently bound protein at the poly(A) end of RF as usually extracted. The extraction procedure includes treatment with phenol-chloroform and with SDS at relatively low salt concentration. However, a CsCl-GuHCl-sarkosyl treatment is necessary to dissociate the non-covalently bound protein. The possible role of this additional protein in polio virus infection remains a subject for further study.

EMC viral RNA has a protein, presumably covalently bound at its 5' end (17). There is a low frequency of circular molecules observed in EMC double-stranded RF preparations (18). Some of the properties of these circular molecules indicate that they are covalently closed (18). However, our observations on circular molecules prompt us to raise the question as to whether the circularization of EMC RF is related to interactions between terminal proteins.

### ABBREVIATIONS

RF: replicative form. RI: replicative intermediate. DNFB: dinitrofluorobenzene. DNP: dinitrophenyl group. BED buffer: 0.125 M  $\text{Na}_2\text{B}_4\text{O}_7$  (pH 9.0), 6.25 mM  $\text{Na}_3\text{EDTA}$  and 10% v/v DMSO. ENTc buffer: 5 mM  $\text{Na}_3\text{EDTA}$ , 0.2 M NaCl, 0.05 M Tricine buffer (pH 8.0). ENP buffer: 10 mM  $\text{Na}_2\text{EDTA}$ , 0.2 M NaCl, 0.1 M NaP buffer (pH 7.2). NaP: sodium phosphate. GuHCl: guanidinium hydrochloride.

### ACKNOWLEDGMENTS

We learned, after initiating this work, that electron microscopic labeling studies of the protein(s) attached to polio RF were independently being carried out by Dr. Jerry Manning and collaborators. Their results will be reported independently.

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